Selective Toxicity of Persian Gulf Sea Cucumber Holothuria Parva on Human Chronic Lymphocytic Leukemia B Lymphocytes by Direct Mitochondrial Targeting

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ABSTRACT: Natural products isolated from marine environment are well known for their pharmacodynamic potential in diversity of disease treatments such as cancer or inflammatory conditions. Sea cucumbers are one of the marine animals of the phylum Echinoderm. Many studies have shown that the sea cucumber contains antioxidants and anti-cancer compounds. Chronic lymphocytic leukemia (CLL) is a disease characterized by the relentless accumulation of $CD5^+$ B lymphocytes. CLL is the most common leukemia in adults, about 25–30% of all leukemias. In this study B lymphocytes and their mitochondria (cancerous and non-cancerous) were obtained from peripheral blood of human subjects and B lymphocyte cytotoxicity assay, and caspase 3 activation along with mitochondrial upstream events of apoptosis signaling including reactive oxygen species (ROS) production, collapse of mitochondrial membrane potential (MMP) and mitochondrial swelling were determined following the addition of *Holothuria parva* extract to both cancerous and non-cancerous B lymphocytes and their mitochondria. Our *in vitro* finding showed that mitochondrial ROS formation, MMP collapse, and mitochondrial swelling and cytochrome c release were significantly ($P < 0.05$) increased after addition of different concentrations of *H. parva* only in cancerous BUT NOT normal non-cancerous mitochondria. Consistently, different concentrations of *H. parva* significantly ($P < 0.05$) increased cytotoxicity and caspase 3 activation only in cancerous BUT NOT normal non-cancerous B lymphocytes. These results showed that *H. parva* methanolic extract has a

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selective mitochondria mediated apoptotic effect on chronic lymphocytic leukemia B lymphocytes hence may be promising in the future anticancer drug development for treatment of CLL. Environ Toxicol 00: 000–000, 2016.
Keywords: sea cucumber; *Holothuria parva*; chronic lymphocytic leukemia; B lymphocyte; isolated mitochondria

INTRODUCTION

Environment has been a source of medicinal products for millennia, with many useful drugs developed from environmental sources. While marine organisms do not have a significant history of use in traditional medicine, the ancient Phoenicians employed a chemical secretion from marine molluscs to produce purple dyes for woolen cloth, and seaweeds have long been used to fertilize the soil. The world's oceans, covering more than 70% of the earth's surface, represent an enormous resource for the discovery of potential chemotherapeutic agents. The systematic investigation of marine environments as sources of novel biologically active agents only began in earnest in the mid-1970s. During the decade from 1977 to 1987, about 2500 new metabolites were reported from a variety of marine organisms and, to keep in perspective (Cragg and Newman, 2013).

The sea cucumber belongs to the Echinoderm phylum. It has been a traditional tonic food in many Asian countries for thousands of years. Many species have been found, among them only several species are edible and of medicinal use (Salarzadeh et al., 2012). The sea cucumbers live on soft sediments, phanerogam sea grass beds and rocky substrata, in depths between 0 and 100 m (Colloca et al., 1997). In the oceans, there are hundreds of varieties of sea cucumbers. Sea cucumbers are famous to exert beneficial effects on human health. Traditionally these echinoderms are used in many Asian countries as a medicine for maintain fitness during long fishing travels or to prevent, reduce or cure several illness like renal deficiency, arthritis or constipation. Several articles published in the last decades came in support to these medicinal purposes showing several biological activities of sea cucumber extracts as antimicrobial, anticancer, and immunomodulatory properties and exhibiting wound healing promoter (Ridzwan et al., 1995; Fredalina et al., 1999; Aminin et al., 2001; Tian et al., 2005).

Chronic lymphocytic leukemia (CLL) is an illness characterized by an accumulation of monoclonal mature B cells in the peripheral blood. Although CLL is the most common leukemia in the Western world, little is known about the biology of the disease (Chiorazzi et al., 2005). Treatment schemes rely heavily on glucocorticoids, chlorambucil, and nucleoside analogs (Hallek et al., 2008). However, despite the initial effectiveness of these drugs in patients with low-grade disease, resistant cells ultimately emerge, leaving no effective treatment options available. It is possible that drug-resistant CLL cells possess intrinsic defect(s) in their ability to undergo apoptosis (Dean et al., 2005). Many investigators have shown that all of natural compounds trigger apoptosis in CLL cells *in vitro* through intrinsic pathway (mitochondria), suggesting that induction of apoptosis may account for their therapeutic efficacy. Mitochondria, the cells powerhouses, are essential for maintaining cell life, and they also play a major role in regulating cell death, which occurs upon permeabilization of their membranes (Green and Reed, 1998; Ravagnan et al., 2002; Breckenridge and Xue, 2004; Green and Kroemer, 2004).Once mitochondrial membrane permeabilization (MMP) occurs, cells die either by apoptosis or necrosis. Key factors regulating MMP include calcium, the cellular redox status (including levels of reactive oxygen species) and the mobilization and targeting to mitochondria of Bcl-2 family members. Contemporary approaches to targeting mitochondria in cancer therapy use strategies that either modulate the action of Bcl-2 family members at the mitochondrial outer membrane or use specific agents that target the mitochondrial inner membrane and the mitochondrial permeability transition (PT) pore (Fagian et al., 1990; Gadelha et al., 1997; Kopustinskiene et al., 2001; Armstrong and Jones, 2002). Activation of the cell death machinery in cancer cells by inhibiting tumour-specific alterations of the mitochondrial metabolism or by stimulating mitochondrial membrane permeabilization could therefore be promising therapeutic approaches (Fulda et al., 2010).

In our study we isolated B lymphocytes from CLL patients and normal lymphocytes from healthy donors. Then we investigated selective toxicity of methanolic extract of *H. parva* on B lymphocytes obtained from CLL patients. Because mitochondria is a major pathway for induction of apoptosis in cells, mitochondrial upstream events such as mitochondrial functionality, mitochondrial swelling, ROS formation, collapse of mitochondrial membrane potential, and finally cytochrome C release were also investigated on mitochondria isolated from B lymphocytes of both CLL patients and healthy donors.

MATERIALS AND METHODS

Sampling

Samples of *H. parva* with helping of divers at depth 6–9 m were collected at Bandar Abbas on the Persian Gulf coast of Iran. The samples put in large containers containing seawater with good aeration and were transferred to laboratory,

Iranian Fisheries Research Institute, Persian Gulf, and Oman Sea Ecological Research Center.

Sample Identification

To ensure the accuracy of the species, a slice of epidermal tissue sample with thickness of 1 mm and area 1 cm^2 were parted and then in a test tube containing 3 mL of liquid bleach was placed. After 20 min, a white precipitate was collected at the end of the tube. A drop of white sediment was spread on a lam and then was observed by light microscopy with lenses 10 and 40. Ossicles with key FAO identification of sea cucumbers for *H. parva* were compared.

Anatomy

Once in the laboratory, the samples were divided into separate components that consist of body wall, respiratory tree, gonad, and digestive tube. Different parts of the *H. parva* body was cut into pieces of 1–2 cm and was kept at 20°C.

Extraction

Samples after dissection, for better performance and durability in a period of 24–48 h were put in freezer-drier device, to make them dry and frozen. The methanol extract was prepared. Dried-frozen samples were transferred to an Erlenmeyer flask containing methanol remained to be extracted for 48 h. Extract by rotarod apparatus, at temperatures $<40°$ were concentrated. After the required time, pure extract was prepared as dry (Salarzadeh et al., 2012).

Standardization of Extract

Total phenols were determined using Ciocalteau's method. Total phenol content was calculated as a gallic acid equivalent using calibration curves prepared with gallic acid standard solutions. Total phenols in samples were 108 ± 7 mg of gallic acid equivalents/100 g. Total flavonoids were determined using aluminum chelating method of Kovacevic. Total flavonoids content was calculated as a rutin equivalents using calibration curves prepared with rutin hydrate standard solutions covering a concentration range between 10 and 50 μg ml^{-1}. The amount of total flavonoids in samples was 31 ± 3.2 mg of rutin equivalents/100 g (Mamelona et al., 2007).

Selection Healthy Donors or Patients with CLL

The 10 CLL patients (6 males and 4 females) aged 58–85 years (with median age of 63 years) were enrolled in this study. CLL was diagnosed and confirmed according to definition of the World Health Organization (WHO) classification by oncologist. Only those patients showing no previous treatments within the last 6 months were included in the present study. All 10 patients were sampled prior to the commencement of any treatment and at least 2 weeks after the transfusion. Age-matched controls were obtained from 10 healthy donors. This study was approved by the Shahid Beheshti University of Medical Science'sethics committee and all the patients and healthy controls signed an informed consent form (Salimi et al., 2015b).

Isolation Lymphocytes

Lymphocytes from venous blood of healthy donors or patients with CLL were prepared by Ficoll–Hypaque density gradient centrifugation. The peripheral blood lymphocytes were maintained in RPMI 1640 supplemented with 10% FCS and used further in the experiments. The percentage of $CD5^+/CD19^+$ (leukemic) cells in the samples from patients with CLL was determined by flow cytometry, using monoclonal mouse anti-human antibodies, CD5 FITC/CD19 PE. It was added stromal cell-derived *factor*−1 to rescue B-lymphocytes from apoptosis Then the cells were counted and cells at density of 10^6/mL were adjusted (Salimi et al., 2015b).

Cytotoxicity Assay

The effect of the Acacetin on lymphocytes obtained from healthy donors or patients with CLL was investigated using MTT assay. Briefly, the cells were seeded in 96-well culture plates at a density of 10,000 cells per well. The acacetin was added to various final concentrations (0, 5, 10, 20, 50, 100, and 200 μM) in triplicates. After 24 h of incubation with acacetin, 25 μL of MTT reaction solution (5 mg mL^{-1} in RPMI 1640 medium) was added to the wells. The optical density was read at 580 nm wavelength in an ELISA plate reader after 4-h incubation of the plates with MTT in an incubator (37°C and 5% CO_2-air). All determinations were confirmed using replication from at least three identical experiments (Liu et al., 1997).

Determination of Caspase-3 Activity

Caspase-3 activity was measured in cell lysate of lymphocytes from different treatments using caspase-3 assay kit ("Sigma's CASP- 3-C)." Briefly, this colorimetric assay is based on the hydrolysis of substrate peptide, Ac- DEVD-pNA, through caspase-3 (Pourahmad et al., 2010a; Salimi et al., 2015b).

Determination of Protein Concentration and Normalized Data

The Bradford assay is very fast for determination of protein concentration. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs. We prepared a standard curve of absorbance versus micrograms protein with albumin (0, 500,

1000, 1500, and 2000 μg mL^{-1}) and determine amounts from the curve. Final concentration of isolated mitochondria was adjusted in 1000 micrograms per milliliter in corresponding buffer (Faizi et al., 2014; Hosseini et al., 2013).

Cell Lysis and Isolation of Mitochondria

Mitochondria were isolated from the lymphocytes by mechanical lysis and differential centrifugation. Briefly, cells were washed with cold PBS at 4°C and centrifuged at 1300 RPM. The pellet was resuspended in cold isolation buffer (75 mmol L^{-1} sucrose, 20 mmol L^{-1} HEPES, 225 mmol L^{-1} mannitol, 0.5 mmol L^{-1} EDTA, pH 7.2), and the cells were disrupted by homogenization. Nonlysed cells and nuclei were spun down by centrifugation at 1000 g for 10 min. The supernatant was further spun at 20,000*g* for 25 min. The pellet, designated as the mitochondrial fraction, was suspended in related buffer. The isolation of mitochondria was determined by measurement of succinate dehydrogenase (Salimi et al., 2015b).

Measurement of Succinate Dehydrogenase

Briefly, mitochondria obtained from patients and healthy donors was suspended in assay buffer (140 mmol L^{-1} KCL, 10 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$, 0.5 mmol L^{-1} KH_2PO_4, 20 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EGTA; adjusted to pH 7.2 with KOH), The assay buffer was supplemented with 1 mg mL^{-1} rotenone and 10 mmol L^{-1} succinate immediately before use. The mitochondria were seeded in 96-well culture plates at a density of 100 μg mL^{-1} per well. The acacetin was added to various final concentrations (0, 5, 10, 20, 50, 100, and 200 μM) in triplicates. After 1 h of incubation with acacetin, 25 μL of MTT reaction solution (0.4% MTT in assay buffer including succinate) was added to the wells. The optical density was read at 580 nm wavelength in an ELISA plate reader after 30-min incubation of the plates with MTT in an incubator. All determinations were confirmed using replication from at least three identical experiments (Pourahmad et al., 2011; Salimi et al., 2015a).

Determination of Mitochondrial Swelling

Purified mitochondria were isolated as described above. Mitochondria suspensions (at 100 Ag protein per well) were incubated in 96-well plates at 25°C in assay buffer (140 mmol L^{-1} KCl, 10 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$, 0.5 mmol L^{-1} KH2PO_4, 20 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EGTA; adjusted to pH 7.2 with KOH) supplemented with 1 mg mL^{-1} rotenone and 10 mmol L^{-1} succinate. Various compounds were added after 10 min of preincubation. The mPTPC inhibitor such as cyclosporine A was added upon initiation of the preincubation period. Mitochondrial swelling was measured spectrophotometrically. This method equates mitochondrial membrane permeability transition with high amplitude swelling of the mitochondria. Mitochondrial swelling results in a decrease in absorbance monitored at 540 nm (Talari et al., 2014).

Determination of the Collapse of Mitochondrial Membrane Potential (MMP)

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. The mitochondrial fractions (1000 μg protein/mL) were incubated with 10 μM of rhodamine 123 in MMP assay buffer (220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 μM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 μM Rotenone). The fluorescence was monitored using Shimadzu RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 and 535 nm, respectively (Pourahmad and O'Brien, 2000; Rezaei et al., 2014).

Determination of Mitochondrial ROS

The mitochondrial ROS measurement was performed using the fluorescent probe DCFH-DA. Briefly, isolated mitochondria from both group of lymphocytes and were placed in respiration buffer (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 μM EGTA, 0.5 mM $MgCl_2$, 0.1 mM KH_2PO_4 and 5 mM sodium succinate) Following this step, DCFH-DA was added (final concentration, 10 μM) to mitochondria and then incubated for 10 min. Then, the fluorescence intensity of DCF was measured using Shimadzu RF-5000U fluorescence spectrophotometer at an excitation wavelength of 488 nm and emission wavelength of 527 nm (Pourahmad et al., 2009; Salimi et al., 2015b).

Determination of Cytochrome C Release

The concentration of cytochrome c was determined through using the Quantikine human Cytochrome c Immunoassay kit provided by R and D Systems (Minneapolis, MN). Briefly, a monoclonal antibody specific for rat/mouse cytochrome c was precoated onto the microplate. Seventy-five μL of conjugate (containing monoclonal antibody specific for cytochrome c conjugated to horseradish peroxidase) and 50 μL of control and test group were added to each well of the microplate. One microgram of protein from each supernatant fraction was added to the sample wells. All of the standards, controls and test were added to two wells of the microplate. After 2 h of incubation, the substrate solution (100 μL) was added to each well and incubated for 30 min. After 100 μL of the stop solution was added to each well; the optical density of each well was determined through the aforementioned microplate spectrophotometer set to 450 nm (Pourahmad et al., 2010b; Rezaei et al., 2014).

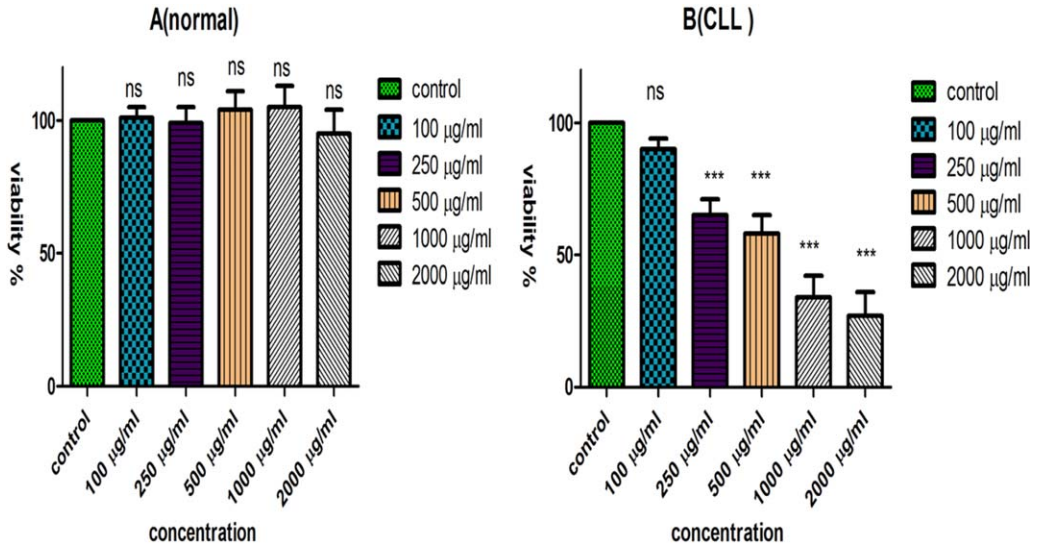

Fig. 1. Cell viability. Effect of H.parva methanolic extract on cell viability in normal and cancerous B lymphocytes. Cells were treated with H.parva methanolic extract and cell viability was measured by MTT assay following 24 h of extract addition. Values were expressed as mean ± SD of three separate determinations (graph A and B). ***: Significant difference in comparison with untreated control ($P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Western Blot Analysis

The treated CLL B-lymphocytes were collected and lysed in a buffer lysis contained 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 2 mM phenylmethyisulfonylfluoride, 0.5% Triton-X as well as protease inhibitor cocktail on ice for 15 min. The protein concentrations of cell lysates were measured with the Bradford method. The proteins were equally loaded on and separated by SDS-PAGE gel, and then transferred to nitrocellulose membranes with moisture transfer technique at 100 V for 2 h. The membranes were blocked by incubation with 5% defatted milk PBS solution for 1 h. After washing, the membranes were incubated in a solution of monoclonal antibodies against human cytochrome c, procaspase-9, caspase 9, procaspase-3, and caspase-3 (in dilution 1:1000) for 1 h, respectively. The rabbit anti-mouse IgG antibodies (1:1000) were used as the secondary antibody. Immunoreactive bands were visualized by the ECL kit (Pierce) and the gray densities were measured with GDS-8000 imaging system (UVP, USA).

Statistical Analysis

Results are presented as mean ± SD. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test when appropriate. Statistical significance was set at $P < 0.05$. And the parameters of mitochondrial dysfunction were analyzed by two way ANOVA and Bonferonie posttest. In all graph were expressed as mean ± SEM and $P < 0.05$ was considered statistically significant.

RESULTS

Viability Assay

Evaluation of sea cucumber H.parva methanolic extract for potential selective toxicity on B lymphocytes cells obtained from CLL patients was carried out using the MTT assay. A 250 µg mL^{-1} concentration of extract reduced 20% of cancerous B lymphocytes viability following 24 h of exposure (Fig. 1 graph B). Toxicity evaluation in normal lymphocytes obtained from healthy donors revealed no significant decrease in cell viability at the same concentration of 250 µg mL^{-1} (Fig. 1 graph A).

Caspase 3 Assay

Caspase-3 is activated in the cell under apoptosis signaling through both extrinsic (death ligand) and intrinsic (mitochondrial) pathways. As shown in Figure 2, *H. parva* methanolic extract significantly increased the activity of apoptosis final mediator, caspae-3 in CLL BUT NOT healthy B-lymphocytes. To figure out the upstream mechanism involved in H.parva methanolic extract induced caspase-3 activation we examined the pretreating effect of Z-IETD a caspase 8 inhibitor and cyclosporine A (Cs.A), an MPT pore sealing agent and Butylated hydroxytoluene (BHT), a ROS scavenger on *H. parva* methanolic extract treated CLL B-lymphocytes. Our results showed that only Cs.A and BHT

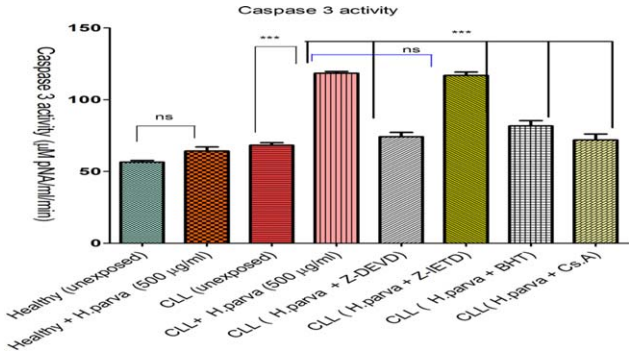

Fig. 2. CLL and healthy B-lymphocytes (10^6 cells mL^{-1}) were incubated in RPMI 1640 medium in conventional condition (37°C and 5% CO_2-air) following the addition of *H. parva* methanolic extract to both groups. As shown in Figure 2, *H. parva* methanolic extract significantly increased the activity of caspae-3 in CLL B-lymphocytes BUT NOT healthy B-lymphocytes. However only Cs.A (5 μM) and BHT (5 μM) but not Z-IETD (10 μM) prevented extract induced caspase 3 activation. Values are expressed as mean ± SD of three separate experiments ($n = 5$). ***: Significant difference in comparison with healthy control ($P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

but not Z-IETD prevented H.parva methanolic extract induced caspase 3 activation ($P < 0.001$) suggesting that *H. parva* methanolic extract activates a ROS-mediated mitochondrial intrinsic pathway in cancerous B-lymphocytes which could end in apoptosis.

Succinate Dehydrogenase Activity

Studying the inhibitory effects of sea cucumber *H. parva* methanolic extract on succinate dehydrogenase activity in the mitochondria obtained from B lymphocytes of both CLL patients and normal healthy donors were carried out by MTT assay. *H. parva* methanolic extract strongly inhibited succinate dehydrogenase activity in a dose-dependent manner in cancerous BUT NOT healthy mitochondria (Fig. 3 graph B, graph A).

ROS Formation Assay

We examined whether the level of ROS in cancerous and normal mitochondria are affected by *H. parva* methanolic extract using DCF-staining. As shown in Figure 4, graph B, *H. parva* methanolic extract treatment at 50, 100, and 200 μg mL^{-1} concentrations for 1 h, significantly induced ROS generation ($P < 0.05$) in cancerous mitochondria. These results suggested that *H. parva* methanolic extract induced ROS generation might underlie its effect on promoting CLL cell apoptosis. However as shown at Figure 4 graph A, treatment with *H. parva* methanolic extract at 50, 100, and 200 μg mL^{-1} concentrations for 1 h, did not induce ROS generation in normal mitochondria. Furthermore, 10 μM cyclosporine A (an MPT blocker) and 50 μM BHT (antioxidant scavenger) strongly inhibited ROS formation induced by *H. parva* in CLL B- lymphocyte mitochondria ($P < 0.05$).

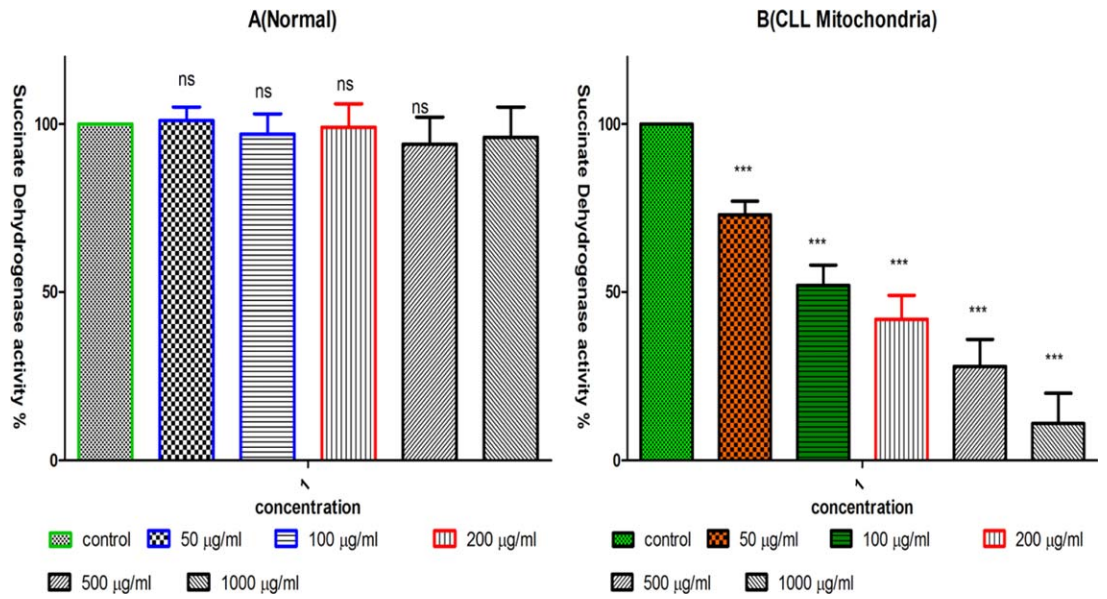

Fig. 3. Effect of *H. parva* methanolic extract on succinate dehydrogenase activity in both normal and cancerous B lymphocytes mitochondria. Mitochondria were treated with *H. parva* methanolic extract and succinate dehydrogenase activity was measured by MTT assay following 1 h of extract exposure. Values are mean ± SD of three separate experiments (graph A and B). ***: Significant difference in comparison with untreated control ($P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

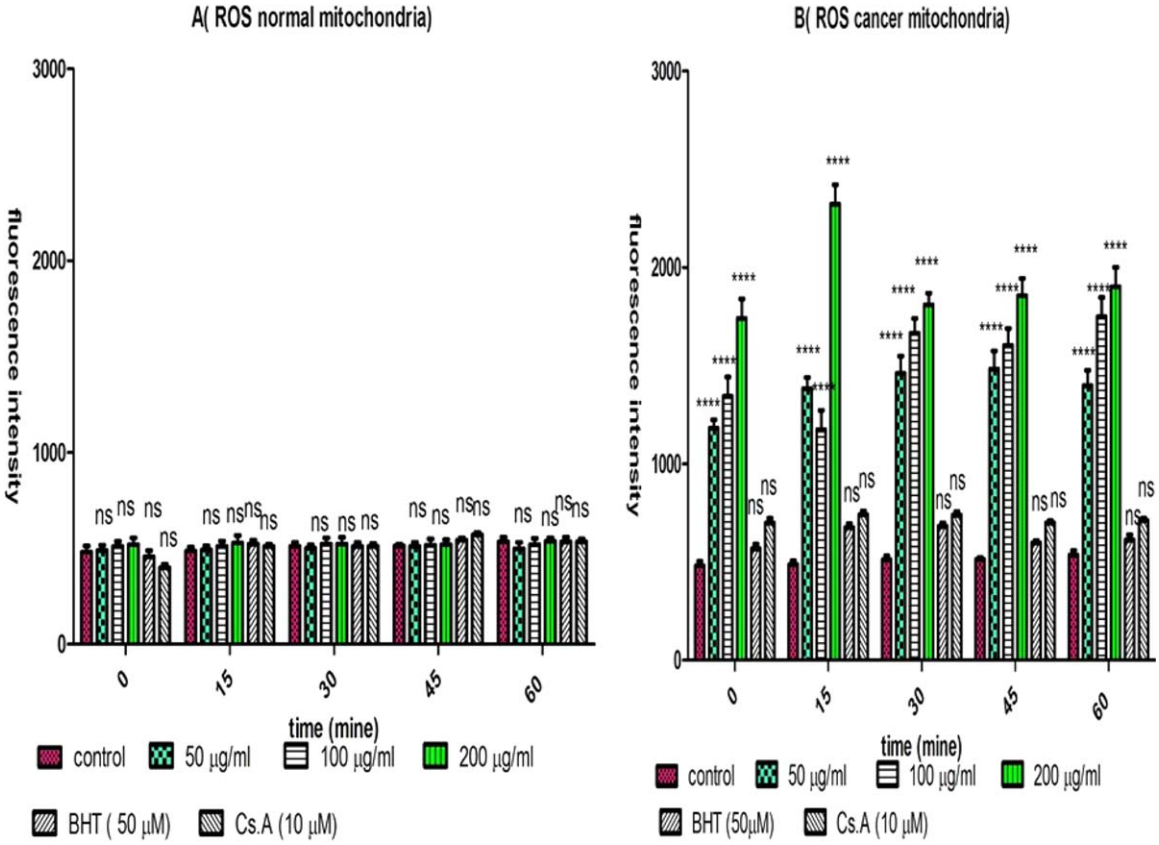

Fig. 4. The effect of *H. parva* methanolic extract on ROS formation in both normal and cancerous mitochondria. Freshly isolated purified mitochondria were obtained from both healthy donors and CLL patients and then incubated with *H. parva* methanolic extract for 1 h. ROS was measured spectroflourimetrically by DCF staining. The mean fluorescence intensity of DCF was enhanced by extract in comparison to control for each group during 1 h of exposure. ****: Significant difference in comparison with untreated control ($P < 0.0001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MMP Assay

To search for the mechanisms involved in apoptosis, we examined the effects of *H. parva* methanolic extract on mitochondrial membrane potential ($\Delta\Psi m$) mitochondria isolated from both groups. Addition of different concentrations of extract (50, 100, and 200 µg mL^{-1}) for 1 h showed decreased $\Delta\Psi m$ in mitochondria obtained from CLL B lymphocytes in comparison to their corresponding untreated control (Fig. 5, graph B). On the other hand, addition of same concentrations of extract (50, 100, and 200 µg mL^{-1}) on normal mitochondria did not show any significant decrease at $\Delta\Psi m$ in comparison to those of their untreated control (Fig. 5, graph A). Furthermore, 10 µM cyclosporine A (an MPT blocker) and 50 µM BHT (antioxidant scavenger) strongly inhibited the decline of MMP induced by *H. parva* in CLL B- lymphocyte mitochondria ($P < 0.05$).

Mitochondrial Swelling

Induction of mitochondrial swelling in isolated lymphocyte mitochondria was monitored by following 540 nm absorbance (A540) decrease. Addition *H. parva* methanolic extract (50, 100, and 200 µg mL^{-1}) resulted in an extensive mitochondrial swelling in cancerous mitochondria obtained from B lymphocytes of CLL patients (Fig. 6, graph B). Addition of same concentrations (50, 100, and 200 µg mL^{-1}) of extract to normal mitochondria did not induce mitochondrial swelling (Fig. 5, graph A).

Cytochrome C

Our results showed that *H. parva* methanolic extract significantly caused mitochondrial swelling and collapse of the mitochondrial membrane potential. These events could result in mitochondrial permeability transition and release of cytochrome c from mitochondria into the incubation buffer. As shown in Figure 7, *H. parva* methanolic extract (100 µg mL^{-1}) induced significant ($P < 0.05$) release of cytochrome c on the liver mitochondria isolated from CLL patients but not normal healthy donors. Significantly, the pretreatment of extract-treated mitochondria with the MPT inhibitor, cyclosporine A (Cs.A) and ROS scavenger, butylated hydroxyl toluene (BHT) prevented cytochrome c

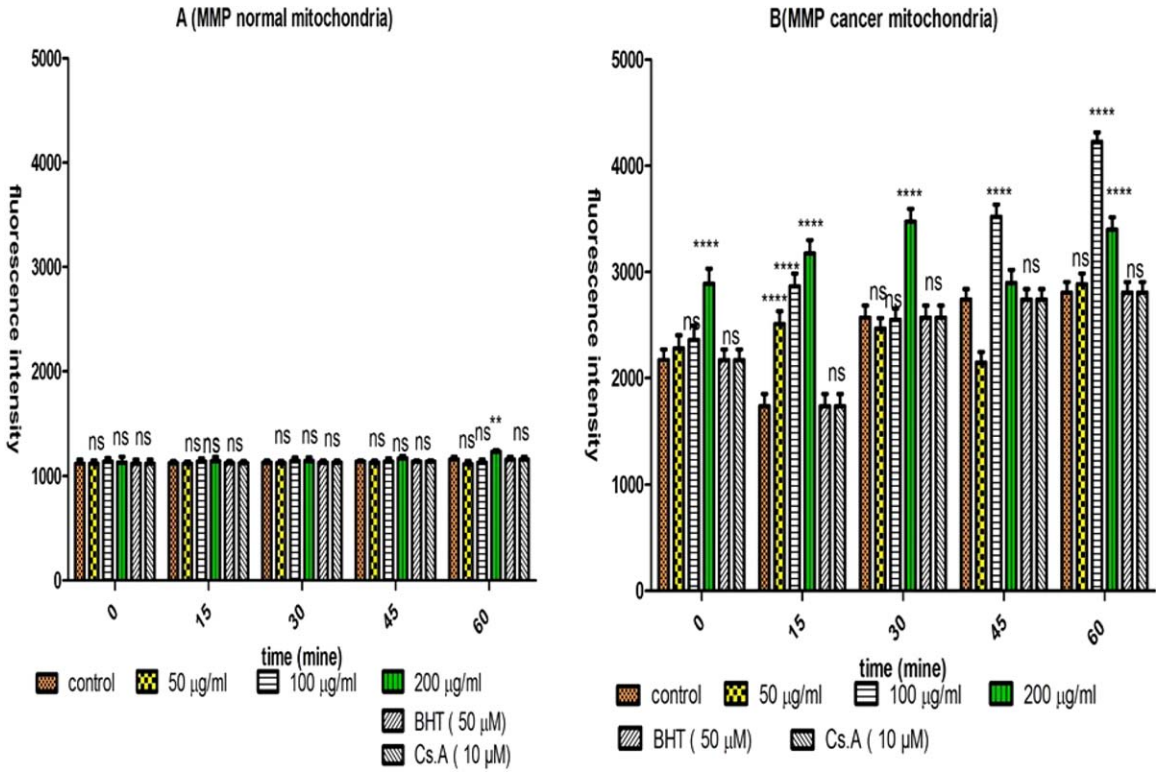

Fig. 5. The effect of *H. parva* methanolic extract on ΔΨm collapse. Freshly isolated purified mitochondria from both healthy and CLL B lymphocytes were treated with different concentrations of extract (50, 100, and 200 µg mL^{-1}) for 1 h. ΔΨm was measured by rhodamine 123 staining with spectrofluorescence method. Our data revealed that *H. parva* methanolic extract could induce a significant decrease in ΔΨm only in cancerous CLL but not in normal mitochondria obtained from healthy donors (graph B, graph A). ****: Significant difference in comparison with untreated control ($P < 0.0001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

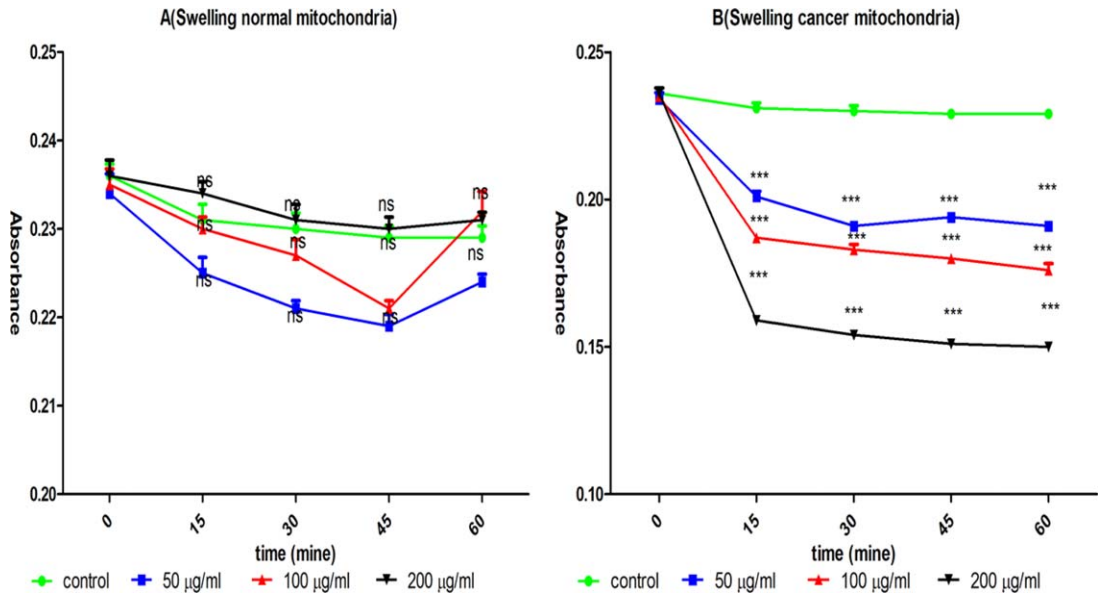

Fig. 6. *H. parva* methanolic extract induced mitochondrial swelling. Addition of *H. parva* methanolic extract (50, 100, and 200 µg mL^{-1}) induced mitochondrial swelling in cancerous BUT NOT normal mitochondria. For swelling evaluation, absorbance of mitochondrial suspensions at 540 nm were monitored every 15 min within 1 h. ***: Significant difference in comparison with untreated control ($P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

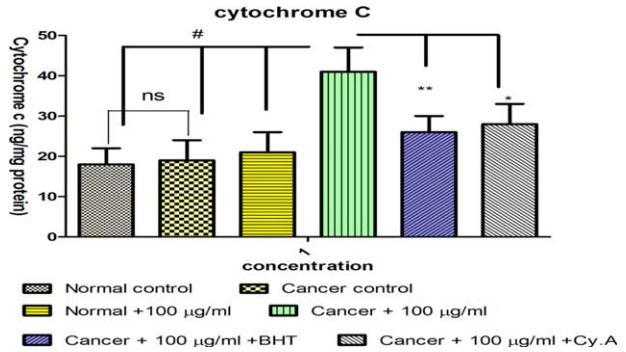

Fig. 7. Effect of the methanolic extract of *H. parva* (100 μg mL^{-1}) on the cytochrome c release in the lymphocyte mitochondria isolated from CLL patients group. As shown in this figure, pretreatment of with BHT or CsA significantly prevented cytochrome c release in the cancerous lymphocyte mitochondria. The amount of expelled cytochrome c from mitochondrial fraction into the suspension buffer was determined by man Cytochrome c ELISA kit as described in above. Values presented as mean ± SD of five separate experiments (n = 5). ∗# Minimal significance level $P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

release as compared with sole *H. parva* methanolic extract-treated group (100 μg mL^{-1}) ($P < 0.05$), indicating the role of oxidative stress and MPT pore opening in extract induced cytochrome c release.

Western Blotting Analysis

To examine whether *H. parva* methanolic extract may induce apoptosis through mitochondrial pathway, we measured the levels of cytochrome c, procaspase 3 and 9 and caspase 3 and 9 of CLL cells after 12 of incubation with *H. parva* methanolic extract. Western blot experiments revealed that, *H. parva* methanolic extract decreased procaspase 3 and 9 expression while that of caspase 3 and 9 were increased (Fig. 8). Also level of cytochrome c in cytosolic fraction markedly increased compared with its level in mitochondrial fraction. These data suggested that *H. parva* methanolic extract induced apoptosis through an intrinsic mitochondrial pathway in CLL cells.

DISCUSSION

Sea cucumber is an important ingredient in traditional medicine and has been considered to be a food among the many communities since ancient times. This traditional science has been further subscribed by scientific studies of its biological potential with biomedical importance (Bordbar et al., 2011). In an attempt to search for new lead natural products with potent biological activities, for the first time we carried out a selective anti-cancer evaluation of the methanolic extract of *Holothuria parva*. Our findings showed selective toxicity of *H. parva* methanolic extract on cancerous B lymphocytes and their mitochondria obtained from CLL patients by MTT assay (Figs. 1 and 3, graph B) while this extract didn't show any significant toxicity effect on normal lymphocytes obtained from healthy donors and their mitochondria (Figs. 1 and 3, graph A). Because the cancer is the second leading worldwide cause of death after cardiovascular diseases, this finding enjoys significant importance (Wijesinghe et al., 2013).

Many anti-tumor agents are reported to exert their anticancer effects by inducing apoptosis of cancer cells (Kamesaki, 1998). Apoptosis is an important biological mechanism that contributes to the maintenance of the integrity of multicellular organisms. Apoptotic cell death can be mediated via several pathways that called extrinsic (receptor) and intrinsic (mitochondria) pathways. Our results proved that *H. parva* methanolic extract could selectively induce apoptosis in CLL but not healthy normal B-lymphocytes and also showed that this selective apoptosis is initiated from mitochondria (Fig. 2). The mitochondrial permeability transition is an important step in induction of cellular apoptosis (Zhang et al., 2012). Many chemotherapeutic agents can target mitochondrial damage, eventually leading to cell death. In addition, many agents induce cellular stress, which may also lead to mitochondrial perturbation and, finally, cell death. A mechanism causing the mitochondrial dysfunction is mitochondrial membrane permeability transition which includes dissipation of the inner membrane potential, osmotic swelling of the matrix, rupture of the outer mitochondrial membrane, release of cytochrome c, and other apoptogenic proteins from the mitochondria, and formation of the

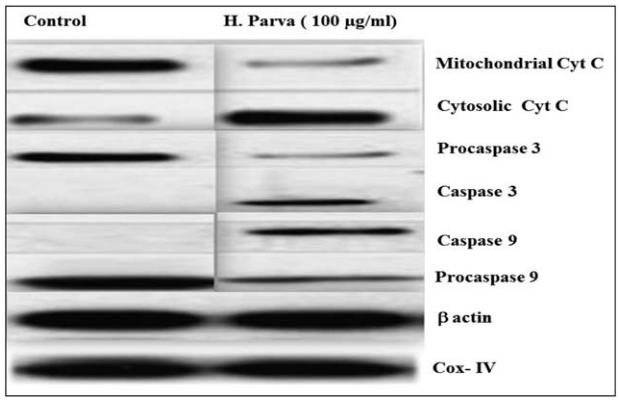

Fig. 8. The effect of H. parva methanolic extract (100 μg mL^{-1}) on expression of cytochrome c, procaspase 3 and 9 and caspase 3 and 9 of CLL B-lymphocytes. CLL B-lymphocytes were treated with *H. parva* methanolic extract for 12 h. At the end of treatment, CLL B-lymphocytes were collected and both the mitochondrial and cytosolic extracts were prepared. Cytochrome c, procaspase 3 and 9, and caspase 3 and 9 were measured by Western blotting. Band 1, untreated CLL B-lymphocytes control; 2, 100 μg mL^{-1} H. parva methanolic extract treated CLL B-lymphocytes.

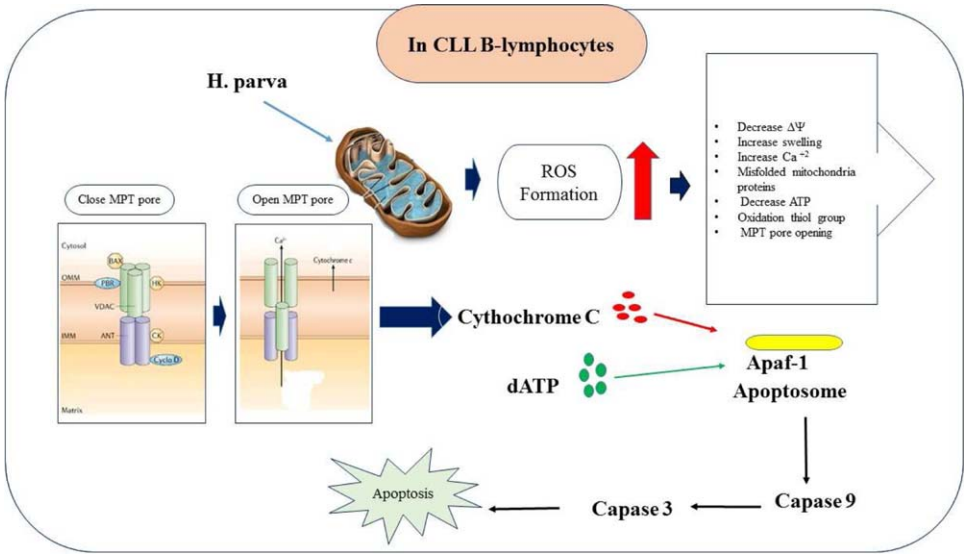

Fig. 9. The schematic representation of *H. parva*-mediated apoptosis action. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

caspase-3 activation complex (Fulda et al., 2010). In this study we showed increased ROS formation after exposure of H. parva extract on cancerous mitochondria while the same effect was NOT observed on normal mitochondria. The mitochondrial respiratory chain is one of the major sources of endogenous ROS (Green and Kroemer, 2004). Several chemotherapeutic agents have been identified that promote mitochondrial ROS generation, for example; mitochondrial electron transport chain modulators (e.g., arsenic trioxide), redox-cycling compounds (e.g., motexafin gadolinium), or disrupt mitochondrial antioxidant defenses, i.e. GSH depleting agents (e.g., buthionine sulfoximine, phenethyl isothiocyanate), and inhibitors of SOD (e.g., 2-methoxyestradiol), and catalase (e.g., 3- amino −1,2,4-triazole) (Pelicano et al., 2003). ROS are responsible for induction of Ca^2 dependent mitochondrial permeability transition (MPT), with swelling of the mitochondrial matrix and rupture of the OMM (Byrne et al., 1999; Maciel et al., 2001). MMP is a universal feature of cell death and is often considered as the "point of no return" in the cascade of events leading to apoptosis (Chipuk et al., 2004; Green and Kroemer, 2004). Our results also revealed collapse mitochondria membrane potential (MMP) only on cancerous mitochondria confirming that MMP occurs after extract treatment on cancerous but not normal mitochondria. Besides, addition of extract to cancerous mitochondria also resulted in mitochondrial swelling while this did not happen on normal mitochondria at same extract concentration. Several mechanisms for the release of Cyt c from mitochondria have so far been proposed in the literature, including dissociation of this peptide from the inner mitochondrial membrane in response to cardiolipin peroxidation by reactive oxygen species (ROS) (Gogvadze et al., 2006). Several researchers have proposed that the release of Cyt c occurs due to the rupture of the mitochondrial outer membrane (Ott et al., 2002). Permeability transition involves the opening of a channel named permeability transition pore complex (MPT pore) (Javadov et al., 2009). In this study we showed release of Cyt c from only cancerous mitochondria after treatment with H. parva extract (100 μg mL^{-1}). Moreover, Cs A and BHT pretreatment completely blocked the extract-induced release of cytochrome c from the cancerous mitochondria which supports the hypothesis that the apoptosis induction via *H. parva* extract is due to an oxidative stress and depends on the opening of the mitochondrial transition pores (Fig. 9).

Finally our results suggest that *H. parva* can act promising as an anticancer drug candidate by directly and selectively targeting mitochobndria in cancerous cells and thus could induce cell death through ROS mediated mitochondrial pathway which finally ends in cytochrome c release, caspase 3 activation and apoptosis in cancerous B-lymphocytes isolated from CLL patients.

The results presented in this article were partly extracted from the thesis of Dr. Maryam Ayatollahi (Pharm.D. graduate of Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences) who performed their theses under the supervision of Prof. Jalal Pourahmad and Prof. Abbasali Motallebi. The investigation was performed in Prof. Jalal Pourahmad's laboratory in the Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. This work was financially supported by a research grant (Grant No: 14603/211) from the Research and Education and Extension Organization (AREEO), Ministry of Jihad-e-Agriculture, Tehran, Iran.

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